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Impaired Wound Contraction in Stromelysin-1–Deficient Mice

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Objective
To determine whether the deletion of stromelysin-1, a single metalloproteinase gene product, will alter the time course and quality of dermal wound repair in mice.

Summary Background Data
After dermal injury, a highly coordinated program of events is initiated by formation of a fibrin clot, followed by migration of keratinocytes, contraction of the dermis, recruitment of inflammatory macrophages, formation of granulation tissue with angiogenesis, and finally tissue remodeling. Matrix metalloproteinases are rapidly induced in the dermis and granulation tissue and at the leading edge of the epidermis in the healing wounds.

Methods
Incisional and circular full-thickness wounds 2 to 10 mm were made in the dermis of stromelysin-1–deficient and wild-type mice. The wounds were analyzed for rate of cellular migration and epithelialization. The wound contraction was examined by immunohistochemical staining for α-smooth muscle actin and fluorescent staining for fibrillar actin.

Results
Independent of the age of the animal, excisional wounds in stromelysin-1–deficient mice failed to contract and healed more slowly than those in wild-type mice. Cellular migration and epithelialization were unaffected in the stromelysin-1–deficient animals. The functional defect in these mice is failure of contraction during the first phase of healing because of inadequate organization of actin-rich stromal fibroblasts.

Conclusions
Excisional dermal wound healing is impaired in mice with a targeted deletion in the stromelysin-1 gene. Incisional wound healing is not affected. These data implicate stromelysin-1 proteolysis during early wound contraction and indicate that stromelysin-1 is crucial for the organization of a multicellular actin network.

Perturbation of the balance between extracellular matrix (ECM) degradation and deposition contributes to a number of pathologic conditions characterized by abnormal healing and chronic inflammation.[1–3] Matrix metalloproteinases are expressed in migrating keratinocytes, the adjacent dermis, and granulation tissue of healing wounds, and their altered functions have been implicated in disease processes characterized by abnormal healing.[4–7]

Stromelysin-1 (matrix metalloproteinase-3) degrades proteoglycans, laminin, fibronectin, the nonhelical domains of collagen types IV and IX, propeptides of type I collagen, and denatured collagens and activates collagenase-1.[8–11] It is synthesized primarily by fibroblasts and to a lesser extent by activated macrophages and keratinocytes adjacent to sites of injury.[12–14] Stromelysin-1 is found in settings where active ECM remodeling occurs, including the stroma of normally healing rabbit corneal wounds,[7] stromal cells and keratinocytes of chronic ulcers,[14] and burn wound fluid from humans.[15] With the possible exception of interstitial collagenase, metalloproteinase activities are overlapping.

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Compensatory mechanisms have been observed in stromelysin-1–deficient (*Str-1*$^{-/-}$) mice in studies of uterine involution[16] and collagen-induced arthritis.[17]

We designed a study to investigate the impact of disruption of the stromelysin-1 gene on incisional and excisional dermal wound healing in mice. In contrast to previous studies on mice with a disrupted stromelysin-1 gene,[16,17] this study describes a phenotype in *Str-1*$^{-/-}$ mice that is not compensated by other metalloproteinases. Our results demonstrate that under the stress of excisional wound repair, deletion of stromelysin-1 results in a failure of wound contraction and significantly delayed healing.

METHODS

Generation of *Str-1*$^{-/-}$ Mice

Str-1$^{-/-}$ mice were generated through gene targeting by homologous recombination in murine embryonic stem cells, as described previously,[16,17] and maintained on 129Sv background. Young (younger than 4 months of age) and old (9 to 16 months of age) wild-type and *Str-1*$^{-/-}$ mice were used in the studies as indicated.

Wounding

All procedures were performed in a laminar animal operating suite under aseptic conditions according to protocols approved by the University of California, San Francisco, Committee on Animal Research. The mice were anesthetized with Metofane general anesthesia (Pitman-Moore, Mundelein, IL). Full-thickness incisional wounds were created on the backs of *Str-1*$^{-/-}$ mice (n = 10 total mice, 28 wounds) and wild-type mice (n = 10 total mice, 29 wounds) and sutured with 5-0 Surgilene (Davis and Geck, Wayne, NJ). Wounds were left exposed to air and harvested after 1, 2, 3, 7, and 10 days. Full-thickness circular excisional wounds 2, 5, 7, and 10 mm in diameter were made on the backs of *Str-1*$^{-/-}$ mice (n = 10 mice, 40 wounds) and wild-type mice (n = 13 mice, 48 wounds) and harvested after 20 days. Wounds were measured (greatest horizontal and vertical diameters) and photographed daily. Full-thickness circular excisional wounds 7 mm in diameter were created and harvested after 12 hours and 1, 2, 3, 5, 7, 10, or 14 days (n = 6 *Str-1*$^{-/-}$ mice, n = 5 wild-type mice; 50 wounds). All wounds were fixed in 10% phosphate-buffered formalin, processed, and sectioned for histologic analysis.

For the actin detection studies, an additional set of 7-mm wounds were created on *Str-1*$^{-/-}$ and wild-type mice (n = 44 wounds), harvested after 4, 12, 24, 48, and 72 hours, fixed, processed, and sectioned transversely and cross-sectionally. At the time of harvest, the mice were killed by Metofane anesthesia and cervical dislocation.

Immunohistochemistry and Staining of Filamentous Actin

Alpha-smooth muscle actin (αSMA) was detected with a mouse antihuman αSMA monclonal antibody (Sigma, St. Louis, MO). The antibody was purified by affinity chromatography using a protein A column (Bio-Rad, Oakland, CA) and biotinylated using the EZ-Link Sulfo-NHS-LC-Biotinylation kit (Pierce, Rockford, IL). Sections were incubated with biotinylated anti-αSMA antibody (40 μg/ml concentration) for 1 hour at room temperature. The biotinylated anti-αSMA antibodies were detected using the avidin–biotin conjugate method (ABC kit; Vector Laboratories, Burlingame, CA). Immunostaining was performed on at least five sections per sample for the following samples: unwounded normal wild-type and *Str-1*$^{-/-}$ skin (n = 4), normal and *Str-1*$^{-/-}$ wounds harvested after 12 hours (n = 4), 1 day (n = 10), 2 days (n = 6), 3 days (n = 4), 5 days (n = 10), 7 days (n = 8), 14 days (n = 12), and 20 days (n = 8). Blood vessels that served as positive controls stained strongly in both *Str-1*$^{-/-}$ mice and control mice. Negative controls (nonimmune goat serum) had no staining.

To detect filamentous actin, cross-sectional tissue samples from *Str-1*$^{-/-}$ and normal wounds harvested at 4 (n = 4), 12 (n = 4), 24 (n = 10), 48 (n = 10), and 72 (n = 8) hours were analyzed. Transverse sections from another set of wounds harvested after 4 (n = 4) and 24 (n = 4) hours were also stained. Sections were incubated overnight at room temperature with FITC-phalloidin (Sigma) in 2.5 μg/ml Tris-buffered saline to detect filamentous actin.[18,19]

Wound Breaking Strength

Incisional wounds (3 cm) were created on the backs of *Str-1*$^{-/-}$ mice (n = 7) and normal wild-type mice (n = 7) and sutured as described above. The mice were killed 10 days after wounding, and three strips of skin (8-mm wide × 30-mm long) were harvested from the wounded area. Breaking strength was tested on a tensometer.[20]

Data Analysis

Mean values and standard errors of the mean were calculated and compared where appropriate with a Student's t test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Full-thickness sutured incisional wounds (10 mm) and full-thickness circular excisional wounds (2, 5, 7, and 10 mm in diameter) were created on the backs of *Str-1*$^{-/-}$ mice and wild-type mice and allowed to heal. The incisional wounds all closed by 72 hours after wounding in both *Str-1*$^{-/-}$ (2.1 ± 0.2 days, 10 mice, 28 wounds) and wild-type mice (1.8 ± 0.2 days, n = 10 mice, 29 wounds). In contrast,

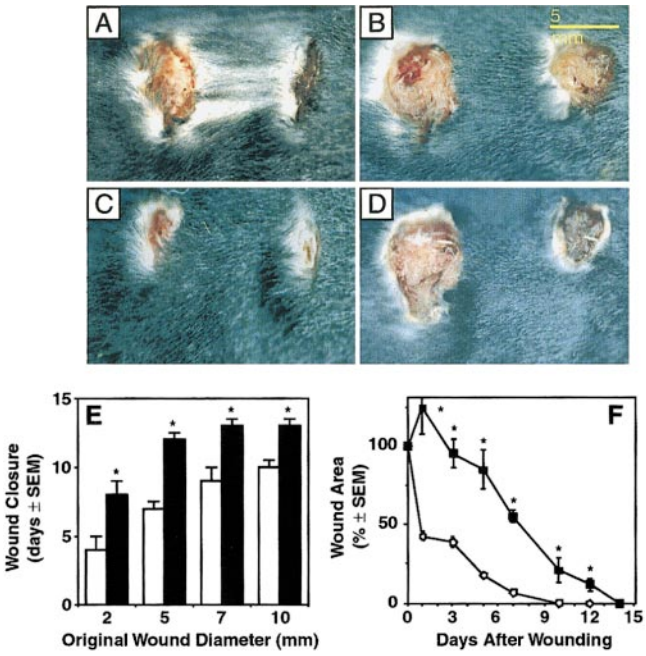

Figure 1. Healing excisional wounds in normal wild-type (A, C) and *Str-1*$^{-/-}$ mice (B, D). In all panels, 7-mm (original diameter) wounds are on the left and 5-mm (original diameter) wounds are on the right. The appearance of wounds 1 day after wounding (A, B) and 7 days after wounding (C, D) is shown. (E) Time for excisional wound closure in wild-type (open bars) and *Str-1*$^{-/-}$ mice (closed bars). (F) Wound areas, expressed as a percentage of the original wound area, in healing excisional wounds (circle, wild-type mice; square, *Str-1*$^{-/-}$ mice). Elliptical wound areas were calculated as $\pi r_1 \times r_2$ = area, where r_1 is the horizontal wound radius and r_2 is the vertical wound radius.

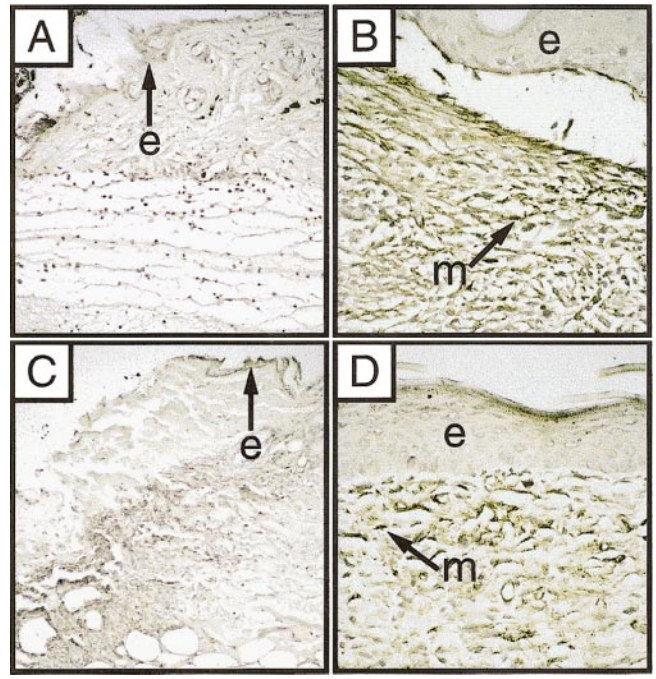

Figure 3. Photomicrograph (×100) of 7-mm excisional wounds from wild-type and *Str-1*$^{-/-}$ mice immunostained for αSMA. Brown pigment marks the myofibroblasts containing αSMA (m, myofibroblast; e, epithelium). One day after wounding, both the wild-type (A) and *Str-1*$^{-/-}$ wounds (C) show few αSMA-containing cells. After 7 days, both wild-type (B) and *Str-1*$^{-/-}$ wounds (D) have abundant myofibroblasts.

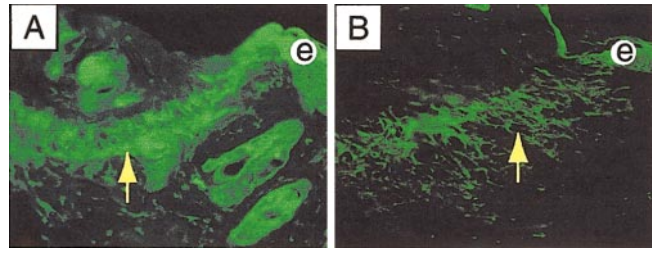

Figure 4. Photomicrograph (×100) of 7-mm excisional wounds stained with FITC-phalloidin. Transverse section from wild-type wounds harvested after 24 hours displays a well-organized actin network at the dermal–granulation tissue interface (A, arrow). Transverse section from *Str-1*$^{-/-}$ wounds harvested after 24 hours displays a loose and disorganized actin network (B, arrow). (e, epithelium)

excisional wounds healed more slowly in the *Str-1*$^{-/-}$ mice than in the wild-type mice (Fig. 1). Time for excisional wound closure did not differ between young *Str-1*$^{-/-}$ mice (younger than 4 months old, n = 4 mice, 16 wounds) and old *Str-1*$^{-/-}$ mice (9 to 16 months old, n = 6 mice, 24 wounds). Macroscopically the excisional wounds in wild-type mice contracted primarily in the cranial-caudal direction during the first 48 hours of healing and then closed at a rate of 50% per day (n = 13 mice, 48 wounds). In contrast, in the *Str-1*$^{-/-}$ mice (n = 10 mice, 40 wounds), excisional wounds did not contract during the first days of healing,

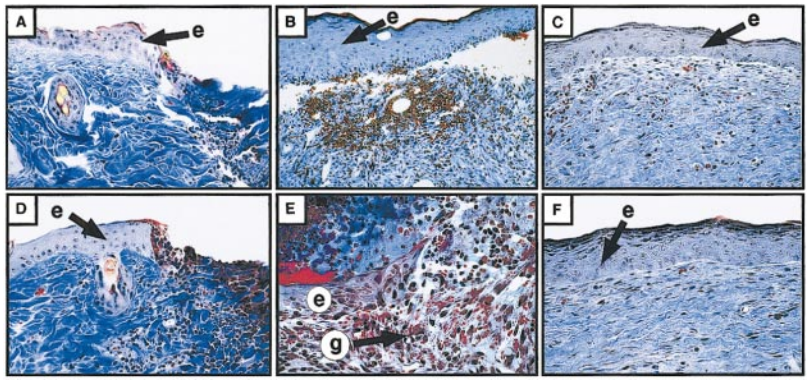

Figure 2. Histologic analysis (×100) of 7-mm excisional wounds from wild-type and *Str-1*$^{-/-}$ mice stained with Mallory's trichrome. After 1 day, both the wild-type (A) and *Str-1*$^{-/-}$ (D) wounds showed a wedge of migrating keratinocytes in the epithelium. After 7 days, the wild-type (B) wounds were microscopically closed, whereas the *Str-1*$^{-/-}$ wounds (E) remained open, although keratinocyte migration appeared histologically normal. After 14 days, both sets of wounds had closed and the dermal and epidermal architecture in the wild-type (C) and *Str-1*$^{-/-}$ (F) wounds appeared normal. (e, epithelium; g, granulation tissue)

showing an initial increase in wound area, followed by a slow diminution as the wounds healed concentrically, with a 5% to 10% decrease in area per day ($p < 0.001$ at each time point, Student's t test).

We next determined the cellular events during the abnormal healing in the mutant mice. Sutured incisional wounds (10 mm) were created on the backs of *Str-1*$^{-/-}$ and wild-type mice and then harvested between 1 and 20 days. Circular excisional wounds 7 mm in diameter were created on a second set of wild-type and *Str-1*$^{-/-}$ mice and then harvested after 4 hours to 14 days. We first determined whether stromelysin-1 was required for keratinocyte migration, as has been found for plasminogen.[21] In the incisional wounds, wedges of migrating keratinocytes appeared in both the *Str-1*$^{-/-}$ wounds and normal wounds within 24 hours. By 72 hours after wounding, all the incisional wounds in both sets of mice displayed microscopic wound closure (defined as complete reepithelialization). Dermal and epidermal architecture of both wounded and unwounded skin appeared histologically normal in the *Str-1*$^{-/-}$ mice. There were no detectable differences in inflammatory infiltrates, granulation tissue, or neovascularization. When incisional wounds harvested from *Str-1*$^{-/-}$ mice and wild-type mice 10 days after wounding were analyzed for wound breaking strength, no difference was detected in mean wound strength between the *Str-1*$^{-/-}$ mice (mean break strength 262 g, $n = 7$ wounds) and the normal mice (mean break strength 255 g, $n = 7$ wounds).

In the excisional wounds, wedges of migrating keratinocytes appeared in both the *Str-1*$^{-/-}$ wounds and normal wounds within 24 hours after wounding (Fig. 2). The microscopic characteristics of the *Str-1*$^{-/-}$ migrating keratinocytes were indistinguishable from those of normal wounds. Although keratinocytes migrated at normal rates in the *Str-1*$^{-/-}$ wounds, the defect in early wound contraction increased the distance over which keratinocytes were required to migrate. Therefore, microscopic wound closure occurred later in the *Str-1*$^{-/-}$ mice (11 ± 1 days, $n = 6$ mice) than in wild-type mice (8 ± 1 days, $n = 5$ mice; $p < 0.01$, Student's t test). Because incisional wounds healed normally in the *Str-1*$^{-/-}$ mice and epithelial cell migration and other aspects of the healing process were normal in the *Str-1*$^{-/-}$ mice, these data suggest that the primary defect involves the inability of the wound to contract.

Two cell types have been implicated in wound contraction, myofibroblasts[22] and dermal fibroblasts.[18,19] Few cells containing αSMA, a marker of myofibroblasts, were detected by immunohistochemistry in either the *Str-1*$^{-/-}$ wounds or the wild-type wounds during the first 4 days of healing (Fig. 3). Cells staining positively for αSMA were detected 7 days after wounding in the dermis of both *Str-1*$^{-/-}$ and wild-type mice but were no longer present in 8/8 wild-type wounds and 7/8 *Str-1*$^{-/-}$ wounds after 14 days. No αSMA containing cells were detectable 20 days after wounding. Thus, there was no defect in formation of αSMA-positive myofibroblasts in the mutant mice.

Early wound contraction may be mediated by formation of organized actin bundles in dermal fibroblasts.[18,19] Because the healing defect in the *Str-1*$^{-/-}$ mice was greatest during the earliest phase of contraction, we analyzed filamentous actin in transverse and cross-sections from *Str-1*$^{-/-}$ and wild-type mice harvested between 4 and 72 hours after wounding by staining with FITC-labeled phalloidin. We observed diffuse actin staining throughout the epidermis in both wild-type and mutant animals at all time points examined. However, an organized network of fibroblasts staining strongly for bundles of actin filaments was observed as a rim at the dermal–granulation tissue interface as early as 4 hours after wounding and persisted through 72 hours in wild-type wounds (Fig. 4). In contrast, loose, disorganized filamentous actin staining without any discernible network structure developed in the fibroblasts in the *Str-1*$^{-/-}$ wounds. These results point to a defect in actin assembly and intercellular organization of fibroblasts to form the multicellular network needed to facilitate wound contraction in the *Str-1*$^{-/-}$ mice.

DISCUSSION

Our data show that stromelysin-1 plays a crucial role in wound healing by modulating early wound contraction. Wound contraction involves interactions between wound fibroblasts and the surrounding extracellular matrix. Two theories have been advanced to explain wound contraction. Myofibroblasts, which are highly contractile and present in granulation tissue, have been implicated as the important cell type.[22–25] Other investigators have argued that the organization of intracellular actin bundles and a rim of organized, densely packed network of fibroblasts may be responsible for initiating the first phase of wound contraction.[18,19,26–28]

We observed that normal wounds contract during the first 48 hours of healing, when few myofibroblasts are present. This indicates that faulty myofibroblast migration or differentiation could not contribute to the mechanism underlying delayed wound contraction in the *Str-1*$^{-/-}$ mice before the fifth day of healing. Our observation that contraction occurred before detection of myofibroblasts in the granulation tissue suggests that although myofibroblasts may be important for long-term wound contraction, scar formation, and matrix remodeling, they are not involved in the first phase of wound contraction. Our data suggest that the organized network of cells containing actin filaments at the edge of the normal wounds may initiate wound contraction. We observed that a tightly woven network of cells containing filamentous actin formed within 4 hours at the junction between the dermis and the granulation tissue in normal healing wounds. In contrast, *Str-1*$^{-/-}$ wounds had loose, disorganized cells staining for actin, without a clearly organized network structure. Stromelysin-1 is necessary for the assembly of this actin network and for the events that allow early wound contraction to proceed.

Fibroblast traction on ECM has been proposed as the mechanism underlying the first phase of wound contraction.[29,30] This requires normal adherence of cells to ECM and normal ECM architecture. The ability of fibroblasts to contract collagen gels *in vitro* depends on cell adhesion receptors interacting with type I collagen and laminin and on cytokines.[31] Contraction of collagen gels also induces collagenase and stromelysin-1.[32] Because the substrates for stromelysin-1 are located throughout the ECM and on cell surfaces, we hypothesize that stromelysin-1 mediates the remodeling of cell–ECM interactions and/or liberation of cytokines or other factors required for organization of a network of actin-containing wound fibroblasts. Stromelysin-1 may also be crucial for modifying the provisional matrix so that it is capable of transmitting the mechanical force necessary for contraction. In the absence of an appropriate scaffold, wound fibroblast migration, attachment and detachment, organization, or traction may be impaired.

Despite the profound defect in wound contraction, all other aspects of healing were normal in the *Str-1*$^{-/-}$ mice. Stromelysin-1 is expressed in basal keratinocytes adjacent to the wound edge but not in the leading edge of migrating keratinocytes in chronic ulcerative wounds.[14] Keratinocyte migration, and thereby wound healing, is compromised in mice lacking plasminogen.[21] Keratinocyte migration and reepithelialization proceeded normally in the *Str-1*$^{-/-}$ mice. Our data support the assertion that stromelysin-1 is not required for active keratinocyte migration or proliferation. Wound breaking strength, which reflects collagen synthesis and long-term ECM remodeling,[20,33,34] was unaffected in the mutant mice, suggesting that stromelysin-1 is not crucial for these processes. Finally, although aging may alter the ECM, resulting in delayed cutaneous wound healing,[33–35] the delayed healing seen in the *Str-1*$^{-/-}$ mice was independent of age. This indicates that the fundamental process requiring stromelysin-1 is unrelated to the age-dependent changes in the ECM. Taken together, these data indicate that stromelysin-1 exerts a unique function early in healing that is critical for initiating wound contraction. It will be interesting to determine whether or when other matrix metalloproteinases expressed during wound healing exert specific functions.

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